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Effect of food level and rearing temperature on burst speed and muscle composition of Western Spadefoot Toad (*Spea hammondii*)

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Summary

1. Past studies on phenotypic plasticity usually focus on ultimate (evolutionary) issues. More recently, proximate (developmental) factors explaining how plasticity is achieved are starting to be addressed.
2. Here, we examine the importance of resource level and temperature on growth rate and burst swimming speed in tadpoles of *Spea hammondii* (Western Spadefoot Toad).
3. Food and temperature manipulations alter growth rate via different developmental processes (cell growth and cell recruitment, respectively) and these processes appear to have consequences for swimming performance in tadpoles.
4. Tadpoles reared at warm temperatures were slower swimmers than those reared at cooler temperatures while food level had no effect on size-specific burst speed. Tadpoles reared at warm temperatures also had more fibres in the tail muscle, probably due to an earlier onset of recruitment. Tadpoles reared at higher food levels had larger muscle fibres, but little differences in fibre number compared to those reared at low food levels.
5. These results indicate that growth by adding cells comes at a performance cost not seen when growth is due to increasing cell size. This developmental difference also has implications for how body size manipulations are carried out in behavioural and ecological studies.

Key-words: Anuran, burst swimming speed

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Introduction

Most prior work on plasticity has dealt with evolutionary issues such as whether or not it is adaptive (Smith-Gill 1983; Doughty & Reznick 2004), whether or not there are genes that promote plasticity (Via *et al*. 1995; Windig, De Kovel & De John 2004), the role of plasticity in diversification (West-Eberhard 2003; Schlichting 2004) and the forms of environmental variation that promote the evolution of adaptive plasticity (Moran 1992). A different approach to the study of plasticity is to focus instead on the underlying developmental mechanisms that cause plasticity (Gilbert 2001; Sultan 2003). Understanding mechanisms can in turn inform us about the ultimate causes (West-Eberhard 2003) and costs (DeWitt, Sih & Wilson 1998) of plasticity. Here, we report on an experimental study of the influence of temperature and resource availability on body size and burst speed in larval amphibians and how these factors are mediated by muscle development. We have chosen to study the joint contribution and possible interaction of temperature and resources because they are known to strongly influence phenotype in a diversity of ectotherms (e.g. Atkinson & Sibly 1996; Gotthard 2004; Angilletta, Steury & Sears 2004).

Larval amphibians are especially likely to encounter variation in temperature and resource availability because they breed in a variety of aquatic habitats. For example, spadefoot toads breed in ephemeral ponds that may vary by as much as 11 °C in temperature among ponds (Morey & Reznick 2004). Resources also vary among ponds depending upon canopy cover (Werner & Glennemeier 1999; Skelly 2004) and tadpole density (Newman 1994; Richter-Boix, Llorente & Montori 2004). Variation in resources and temperature have profound but distinct effects on growth and development rates. Tadpoles raised with more food grow faster, metamorphose earlier and at a *larger* size than those raised with less food (Fig. 1a). Tadpoles reared at warmer temperatures grow faster and metamorphose earlier, but at a *smaller* size than those reared at cooler temperatures (Fig. 1b). Differences in growth trajectories must derive from differences in how bodies are constructed

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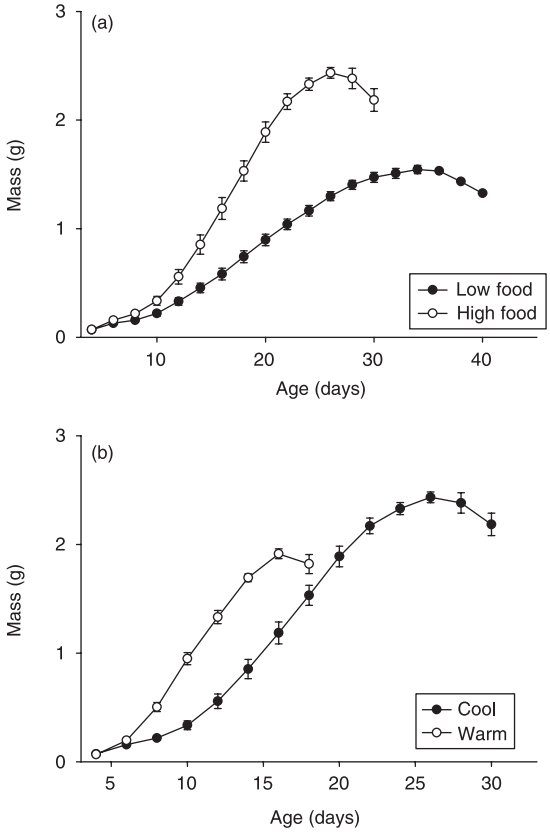

Fig. 1. Growth trajectories of *Spea hammondii* reared at (a) two different food levels and (b) two different temperatures. Points are means (± 1 SE) of five tadpoles each from two broods weighed every other day.

under these conditions. In *Drosophila melanogaster* variation in adult body size is determined primarily by cell number when food level is manipulated (Robertson 1959; De Moed, D Jong & Scharloo 1997), but cell size when rearing temperature is manipulated (Robertson 1959; Partridge *et al*. 1994; Azevedo, French & Partridge 2002). In vertebrates the largest single tissue is skeletal muscle making up 40% of wet weight in most mammals (Vogel 2001) and up to 80% in some fish (Weatherley & Gill 1987). It seems likely therefore that growth patterns of muscle will reflect growth patterns of the whole body in vertebrates. Feeding rate is known to alter muscle fibre number in some teleosts (Galloway, Kjorsvik & Kryvi 1999; Johnston *et al*. 2002) while rearing temperature can alter both fibre number and size (reviewed in Johnston & Hall 2004).

The different developmental mechanisms underlying plasticity in growth are likely to translate into differences in performance. For example, tadpoles of several anuran species reared at warm temperatures are typically slower swimmers than those reared at cool temperatures (*Bombina orientalis* Parichy & Kaplan 1995; *Limnodynastes peronii* Wilson & Franklin 1999; *Xenopus laevis* Wilson, James & Johnston 2000; *Hyla regilla* Watkins 2000). A similar pattern has also been shown in a fish, the spring-spawning herring (Johnston, Vieira & Temple 2001). Whether or not these differences can be linked to muscle composition is not known (although Watkins 2000 linked rearing temperature to differences in myofibrillar ATPase activity). To our knowledge, the effect of feeding level on burst speed has not been tested in tadpoles (but see Billerbeck *et al*. 2001 for an example in fish).

Here, we examine the effects of food and temperature manipulations on swimming speed in *Spea hammondii* (Western Spadefoot) tadpoles and whether or not differences correlate with muscle composition (fibre size, fibre number and extracellular matrix). Given the current state of the field, it is hard to make specific predictions about how rearing temperature and food level will influence either swimming speed or muscle composition. The goal of our study was to determine correlations among these factors. Specifically, we wanted to know: (1) if *S. hammondii* tadpoles reared at warm temperatures are slower swimmers than those reared at cool temperatures as has been found in other tadpoles, (2) if tadpoles fed large daily rations are slower swimmers than those on small rations as has been seen in some fish, (3) how does muscle composition depend upon these environmental manipulations, and (4) how does environmentally induced variation in muscle composition correlate with swimming speed?

Materials and methods

Egg masses of *Spea hammondii* were collected from two populations in western Riverside county, California, in February 2003. Hatchlings from a given egg mass were kept in 4-l plastic shoe-box containers and fed *ad libitum* once per day. Four days after hatching, tadpoles were blotted dry, weighed to 0·001 g and placed individually into 2-l containers. Containers were placed either on shelves and kept at room temperature (20·5–21·5 °C) or in wading pools filled with water warmed by aquarium heaters (24·5–26·5 °C). These temperatures were chosen because they fall within the range this species experiences in the wild (Morey & Reznick 2004) and a difference of 4–5 °C matches what we found between adjacent breeding pools in one of the populations. Half of the tadpoles in each temperature treatment were placed on a low food regimen (0·020–0·025 g of food per day) and half on a high food regimen (0·095–0·100 g per day). These food levels were chosen because they give growth rates at the warmer temperature that match those normally seen in the field (Morey & Reznick 2004). Food was a 3:1 ratio of ground rabbit chow and fish flake food. Seventy-five tadpoles were used per treatment except for the low food, warm treatment where only 40 tadpoles were used (all from a single population). Tadpoles were kept on a 13:11 h light : dark cycle and water changed every 2–3 days.

Swimming trials were conducted 6 days later (10 days posthatch). This time-frame constitutes one-half to one-third of the larval period depending upon treatment (Fig. 1). Trials were conducted in a 30 cm × 30 cm aquarium with a water depth of 2 cm to minimize

vertical movement. Because of room constraints all trials were conducted at room temperature (20·5–21·5 °C). The tadpole was allowed to acclimate for 1 min then positioned so it faced away from the wall of the aquarium and tapped on the tail with a blunt probe. This initiated a startle response and the tadpole would usually swim for 1–2 s. Swimming trials were videotaped at 30 frames/s in a mirror placed at a 45° angle below the aquarium. After 3–4 swims the tadpole was killed in MS-222, blotted dry, weighed and preserved in 10% neutrally buffered formalin. Burst speed was estimated by measuring the most anterior point on the head on each successive frame. Burst responses of tadpoles tend to slow after the first half second (Arendt 2003), so speed was estimated as the sum of the distance travelled between each frame for the first 15 frames after movement started.

Muscle composition was determined for tadpoles used in swimming trials as well as a similar set of tadpoles maintained on treatments for an additional 6 days (near metamorphic climax for the high-food, warm treatment). Tail muscle composition was measured by removing the tail at its base and embedding it in paraplast. Ten micrometre cross-sections of the tail were cut and stained with haematoxylin and eosin. Photomicrographs of cross-sections were transferred to a computer and measured using SigmaScan Pro 5 software. Mean fibre area was estimated for the muscle on one side of the tail by measuring the long and short axes of a fibre and assuming area could be approximated by an oval. Care was taken to ensure that fibres were in cross-section, which are easily distinguished from oblique cuts. We measured all fibres we could clearly see (mean of 225 per tadpole, range 150–322 fibres). A layer of small and difficult to measure fibres lies just below the epidermis of the tail. Areas of these fibres were not estimated, but they were included in the count of fibre number. Amount of extracellular matrix was estimated as ECM = [(total muscle area) – (mean fibre area) ∗ (fibre number)]/total muscle area.

STATISTICAL ANALYSIS

Body mass was ln-transformed for all analyses and growth rate estimated as $\ln(mass_2/mass_1)$/time. Average and maximum (i.e. fastest of the 3–4 swims) burst swimming speed for a given tadpole give similar results, so only average burst speed is reported here. The effect of food level and temperature on swimming speed was determined using analysis of covariance with ln(mass) as a covariate. Developmental stage (following Gosner 1960) was also tested as a covariate, but was never found to be significant so is not considered further.

Cross-sectional area of muscle fibres were ln-transformed to minimize heteroscedasticity and mean fibre area and ECM estimated from the transformed distributions. The effects of food level and temperature on muscle composition (mean area, fibre number and ECM) were analysed using a multivariate analysis of covariance with ln(mass) as a covariate. Direct effects of each factor on muscle composition were determined from the canonical variates generated by the MANCOVA. Finally, because results indicate temperature has an important effect on muscle fibre recruitment, we examined muscle fibre number across treatments at two ages, at 10 days when swimming trials were conducted and on a group of tadpoles raised in parallel and collected 6 days later.

Results

There were no significant differences between populations ($F_{1,245} = 0{\cdot}46$; $P = 0{\cdot}50$) so they were combined for all further analysis. Rearing temperature had a significant effect on burst swimming speed ($F_{1,245} = 9{\cdot}73$; $P = 0{\cdot}002$) with warm-reared tadpoles being slower than cold-reared tadpoles (Fig. 2). Warm reared tadpoles average a speed of 17·3 cm/s (adjusted least squares mean) while cold-reared tadpoles averaged 18·5 cm/s. Feeding level had no effect on burst speed after accounting for body size ($F_{1,245} = 0{\cdot}08$; $P = 0{\cdot}8$) and there was no interaction between treatments ($F_{1,245} = 2{\cdot}15$; $P = 0{\cdot}14$).

Multivariate effects on muscle composition were significant for both temperature (Wilk's $\lambda = 0{\cdot}97$; $P = 0{\cdot}046$) and food (Wilk's $\lambda = 0{\cdot}87$; $P < 0{\cdot}001$) with a non-significant interaction (Wilk's $\lambda = 0{\cdot}99$; $P = 0{\cdot}35$). Influence of environmental variables on muscle composition were further examined by looking at loadings on canonical variates. Fibre number had the highest loading on the canonical variate for temperature (Table 1) and this was the only significant variable in the univariate analysis. Adjusted least square means for fibre number in cold-reared tadpoles was 427 ± 5 fibres (mean ± SE) while warm-reared tadpoles averaged 450 ± 6. Fibre size had the highest loading on the canonical variate for food level (Table 1) and was the only significant variable in the univariate analysis.

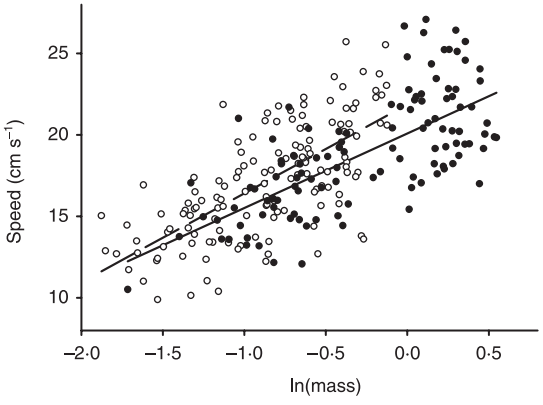

Fig. 2. Relationship between burst swimming speed and body mass for 10-day-old tadpoles reared at warm temperatures (filled symbols, solid line) and cool temperatures (open symbols, dashed regression line). Slopes of regressions do not differ significantly but the regression for cool-reared tadpoles has a significantly lower intercept.

Table 1. Loadings of muscle characteristics on canonical variates derived from MANCOVA. The temperature–food interaction was not significant and so is not shown here

Source	Temperature	Food	ln(mass)
Fibre area	−0·060	+0·849*	+0·924*
Fibre number	+0·849*	−0·203	+0·220*
ECM	+0·214	−0·159	−0·435*

*Factor(s) significant in univariate analyses.

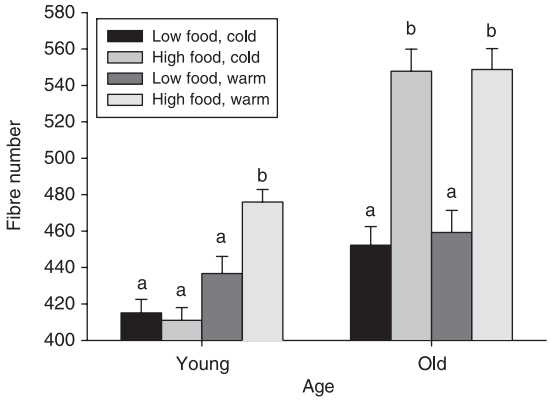

Fig. 3. Muscle fibre number for each treatment at the age where swimming trials were conducted (young) and 6 days later (old) (mean ± 1 SE). At the test age fibre number does not differ between the two cold treatments (left bars) while the warm treatment (right bars) have already started to recruit new fibres. Because fibre number increases in all treatments at the later age, the second phase of fibre recruitment must begin for the cold treatments sometime between when samples were taken. Bars with different letters are significantly different from each other (Tukey *post hoc* test) within an age class.

Adjusted least square means for muscle fibre areas were 6·980 ± 0·032 for low-fed tadpoles and 7·200 ± 0·025 for high-fed tadpoles (ln-transformed values). Fibre size also had the largest loading on ln(mass) (Table 1), although all three variables were significant in the univariate analysis.

For 10-day-old tadpoles (when swimming trials were conducted), increasing food led to increased fibre recruitment ($F_{1,236} = 5{\cdot}1$, $P = 0{\cdot}025$), but only for the warm-reared tadpoles (temperature × food $F_{1,236} = 7{\cdot}8$, $P < 0{\cdot}01$; Fig. 3, left). Warm-reared tadpoles also had more fibres ($F_{1,236} = 30{\cdot}1$, $P < 0{\cdot}001$). Fibre number increased in all treatments between day 10 and day 16 and although the food effect is still strong ($F_{1,145} = 51{\cdot}0$, $P < 0{\cdot}001$), temperature effects ($F_{1,145} = 0{\cdot}1$, $P = 0{\cdot}8$) and interactions ($F_{1,145} = 0{\cdot}1$, $P = 0{\cdot}8$) have disappeared (Fig. 3, right).

Discussion

Tadpoles reared at warm temperatures are slower swimmers than those reared at cool temperatures, a finding in agreement with previous work on other anuran species (Parichy & Kaplan 1995; Wilson & Franklin 1999; Wilson *et al*. 2000; Watkins 2000) as well as in larval herring (Johnston *et al*. 2001). We found no effect of feeding level on swimming speed after adjusting for body size. To our knowledge, the effect of food level on swimming speed in tadpoles has not been examined in the past.

Tadpoles reared at warmer temperatures also have more fibres in their tail muscles than do cold-reared tadpoles. Johnston *et al*. (1998) found that larval spring-spawning herring incubated at 8 °C had more and larger muscle fibres than those reared at 5 °C. Using a different cohort, Johnston *et al*. (2001) found that herring incubated at 10 °C were slower swimmers than those incubated at 5 °C. The greater muscle fibre number in warm-reared but slower swimming herring matches what we found for *S. hammondii* tadpoles, although we found no effect of temperature on fibre size. This discrepancy probably reflects our use of body size as a covariate with fibre size which Johnston *et al*. (1998) did not. When we remove body size from our analysis we also get a highly significant effect of temperature on muscle fibre size. Thus our results for *S. hammondii* match the overall pattern identified by Johnston *et al*. for herring. Although food level had no effect on swimming speed once we controlled for body size, it did have a significant effect on muscle composition, especially muscle fibre size. One reason that this difference in muscle composition did not translate into differences in swimming performance may be that it parallels normal size differences in muscle composition.

Why would an increase in fibre number make warm-reared tadpoles slower swimmers? We suspect that this relates to the way in which temperature influences the underlying pattern of new fibre recruitment during muscle growth. In teleosts there are three phases of fibre recruitment to the lateral muscle (Alami-Durante *et al*. 2000; Johnston *et al*. 2000). First, an initial population of muscle fibres is derived from the somites in the embryo. Second, fibres are recruited to the periphery from adjacent mesenchymal tissue in the late embryo or larva. Finally, fibres are recruited from satellite cells throughout the muscle during juvenile growth. We have found at least two phases of fibre recruitment in the tail muscle of *Spea hammondii*. At 10 days posthatch, when swimming trials were conducted, the warm-reared tadpoles have started the second phase of recruitment while the cold-reared tadpoles have not (Fig. 3). Similarly, Stoiber *et al*. (2002) show evidence that the second phase of recruitment begins earlier in warm-reared Danube bleak. An earlier onset of the second phase of recruitment means that, at a given size, tail muscles of warm-reared tadpoles will consist of more fibres that are younger and smaller than cold-reared tadpoles. This would usually lead to a decrease in mean fibre size. The reason we failed to detect an effect of temperature on fibre size may be because temperature also accelerates growth of fibres that are already present, at least at high food levels. To test this, we looked

at how temperature treatments differed in the variance in fibre sizes within a single tadpole. Warm-reared tadpoles do have a greater variance in fibre size within their muscles (cool = 3·2, warm = 3·5; $F_{1,244} = 6{\cdot}5$, $P = 0{\cdot}011$) as one would expect if temperature influences fibre growth.

Our results for burst speed match those seen in other anuran tadpoles. However, some caution may be warranted in interpreting the reduced swimming speed of warm-reared tadpoles. We tested swimming speed at the cooler temperature and gave the warm-reared tadpoles just 1 min to acclimate to the temperature. Although this should bias results in the direction we found, we do not think the slower swimming speed of warm-reared tadpoles is due to lack of acclimation time. First, the temperature difference was just 4 °C. In the wild, breeding ponds vary by as much as 15 °C throughout the day and as much as 5 °C between the shallow and deeper areas within a pool (Morey & Reznick 2004). In addition, all warm-reared tadpoles experienced the cooler temperature prior to 4 days posthatch when the treatments were set up and twice afterwards during water changes. Although acclimation time cannot be entirely ruled out as an explanation for the results we present here, we think it more likely that differences in muscle composition explain differences in swimming performance. Swimming tests at multiple temperatures will be needed to confirm this assertion.

Although the plastic response to both increased resources and increased temperature is to accelerate growth, it seems clear that different developmental mechanisms drive this response. Warmer temperatures accelerate the onset of secondary muscle fibre recruitment in *S. hammondii*. As a result, warm-reared tadpoles appear to have a disproportionate number of small, immature muscle fibres. Although this will increase growth rate (Arendt 2000), it may also lead to decreased burst swimming speed because smaller fibres provide less force overall (Vogel 2001). Increased food levels result in faster growth primarily by increasing growth of muscle fibres, a process that does not seem to affect size-specific burst speed. However, a direct test of how fibre size contributes to burst speed is still needed. One implication of these results for evolutionary studies of growth rate is that one mechanism, cell recruitment, carries a performance cost while the other, cell growth, does not. This cost may be worth paying in some circumstances because cell recruitment has the potential to produce faster growth rates than does cell growth. In addition, experimental studies of behavioural and ecological interactions often manipulate body size either by manipulating resources or temperature. Our results indicate how one manipulates body size will influence individual performance and thus how individuals interact. Caution must be taken to use the manipulation that is most relevant to the system under study, and not to try to directly compare studies using different manipulations.

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